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14. ABSTRACT

We have developed a 'knock-in' mouse model with a mutation (glycine 525 to leucine, G525L) in estrogen receptor alpha (ERα) that permits exogenous regulation of its ligand-induced signaling pathways, while not affecting ligand-independent signaling. The G525L ligand-binding pocket mutation significantly reduces ERα response to endogenous estrogens. These female estrogen non-responsive ERα knock-in (ENERKI) mice had immature and hypoplastic uterine and vaginal tissues and only developed rudimentary mammary gland ductal trees. Ovarian tissues contained no corpora lutea, indicating these mice are infertile due to anovulation. In addition, 89% of the ovaries contained large, hemorrhagic, cystic follicles. This physiology is consistent with a lack of estrogen negative feedback at the pituitary, which results in chronically elevated circulating levels of luteinizing hormone (LH). These phenotypes were similar to those of the ERα knock-out (αERKO) mice, confirming ligand-induced activation of ERα is important in female reproductive tract development. Although the G525L mutation significantly reduces ERα response to endogenous estrogens, the ERα selective agonist propyl pyrazole triol (PPT) was still able to activate the mutant ERα in uterotrophic assays in the ENERKI females. Therefore, ERα signaling pathways can be regulated in developing mice as well as in adult animals with genetically induced mammary cancers through PPT administration or withdrawal.

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estrogen receptor, steroid receptor, endocrine signaling, estrogen

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INTRODUCTION

Estrogen receptor alpha (ERα) is a crucial therapeutic target for hormone dependent breast cancers. More effective treatment and prevention strategies are likely to emerge from an improved understanding of ERα mechanisms *in vivo*. To achieve this goal, we have developed a 'knock-in' mouse model with a mutation in ERα (glycine 525 to leucine, G525L) that permits exogenous regulation of its ligand-induced signaling pathways, while not affecting ligand-independent signaling. In these estrogen non-responsive ERα knock-in (ENERKI) mice, the ligand-binding pocket mutation significantly reduces ERα response to endogenous estrogens but not to the synthetic nonsteroidal ERα selective agonist propyl pyrazole triol (PPT). Therefore, ERα signaling pathways can be regulated in these mice through PPT administration or withdrawal. These activities can be regulated both in developing animals as well as in adult animals with genetically induced mammary cancers, providing valuable information about the role of ERα in mammary gland development and carcinogenesis.

BODY

Task 1: To define the contribution of classical ERα activation in murine mammary gland development.

1. Analyze the mutant G525L ERα knock-in mouse phenotype

a. Genomic DNA from ENERKI animals was sequenced and confirmed the G525L mutation was present. A reverse transcriptase polymerase chain reaction (RT-PCR) strategy was developed to confirm transgene expression. RT-PCR can be used to quantify mRNA levels from smaller samples than an RNase protection assay and should provide better results. The primers have been developed and tested on wild type RNA, but technical difficulties were encountered with the uterine samples. Therefore, we sent the samples to our collaborators at Northwestern University, since they routinely perform RT-PCR, and we should get the results soon. Uterine ERα protein levels were analyzed by Western blots (Figure 1A). There were equivalent levels of ERα in the wild type, heterozygous, and ENERKI uterine tissues. In addition, mutant G525L ERα was detected in heterozygous and ENERKI uterine tissues with the 6xHis-tag antibody (Figure 1B). Immunohistochemistry (IHC) confirmed these results. ENERKI ERα protein levels were equal to wild type and heterozygous levels in the heart, kidney, liver, lung, mammary gland, preputial gland, spleen, and vagina, and were slightly lower in the ovary.

b. To evaluate the phenotype of all potential sites of mutant G525L ERα expression, reproductive tissues from 6-, 12-, and 20-week-old female mice were analyzed. Luciferase assays with Ishikawa cells, cotransfected with mutant G525L ERα and an estrogen response element (ERE)-reporter, showed transcription was not stimulated by low concentrations of E2, but was stimulated by DES or genistein (Figure 2). Since genistein is a phytoestrogen and may stimulate the mutant G525L ERα *in vivo*, half the animals were placed on a soy-free diet. ENERKI mice were significantly larger than

their wild type and heterozygous littermates by 12 weeks of age (Table 1). Body weight was increased by 19% and 23%, in homozygous females on a regular and soy-free diet, respectively. This was partly due to a significant increase in gonadal and mammary fat pad weights in females on a soy-free diet (Table 1). This increase may be due to the loss of phytoestrogen-induced mutant ERα activation. These results, combined with the similar phenotype of ERα knock-out (αERKO) females[1], support the idea that ligand-induced ERα signaling is important in regulation of female adiposity. Whether this phenotype is due to changes in regulation of metabolism, reduced physical activity, or increased appetite, remains to be determined.

ENERKI reproductive tract phenotypes on a regular and soy-free diet were identical. ENERKI uteri were significantly smaller than their wild type and heterozygous littermates (Figure 3A-C). The average uterine wet weights (reported as uterine wet weight/body weight ratio x 100) were 0.39 +/- 0.05, 0.46 +/- 0.04, and 0.07 +/- 0.01 for wild type, heterozygous, and homozygous females, respectively. The homozygous uterine tracts developed normally and possessed luminal and glandular epithelium, evidence of vascularization, and endometrial stroma (Figure 3D-F). But, they were also severely hypoplasic and completely lacked an edematous stroma (Figure 3C). Thus, the histological evidence supports the finding that the overall wet weigh of the uteri was significantly lower in homozygous animals. In addition, the presence of granular, stratified luminal epithelium containing large secretory vacuoles usually present in ovulating animals was lacking in the homozygous mice (Figure 3G-I). Luminal epithelium of heterozygous animals appeared to have a high cytoplasmic content with less visible vacuoles (Figure 3H). ENERKI vaginal tissues were also severely hypoplastic and lacked cornification. Homozygous preputial (clitoral) glands were masculinized and 10 times larger than those of wild type and heterozygous females.

Continuous mating studies confirmed the ENERKI females were infertile. The ovaries of all three genotypes displayed follicles ranging from primordial to developed antral follicles (Figure 4A-C). The ovaries also had no developmental abnormalities in the ovarian surface epithelium or the oocytes. Ovaries from ENERKI animals in general had a higher number of antral follicles with the majority containing pyknotic nuclei consistent with atresia (Figure 4C). The higher number of developed antral follicles is likely due to the lack of corpora lutea formation obvious in ENERKI animals (Figure 4C). These data indicate that ENERKI mice are likely infertile due to anovulation. Theca cells that respond to luteinizing hormone (LH) by proliferating and producing androgens became hypertrophied in some of the heterozygous and all of the ENERKI female mice (Figure 4D-F). The lack negative feedback by estrogen at the level of the hypothalamus would result in chronic stimulation of these cells. LH levels were significantly higher in the ENERKI females (Table 2). The stromal cells in the ENERKI animal demonstrated signs of luteinized interstitial cell hyperplasia where the cells acquire a high cytoplasmic to nuclear ratio reminiscent of the cells of the corpora lutea (Figure 4G-I). Unique to the ENERKI animals were the presence of hemorrhagic cysts, apparent in 89% of the analyzed animals, which appeared to develop from atretic antral follicles (Figure 4C). Finally, the appearance of granulosa cells not encapsulated into follicles in ENERKI animals was detected in all of the ovaries analyzed but did not progress at the 12 week

age to granulosa cell tumors (Figure 4C). ENERKI female LH levels were elevated 12-fold over wild type levels (Table 2). Heterozygous and ENERKI E2 levels were 2- and 3.5-fold higher than wild type levels, respectively (Table 2). ENERKI testosterone levels were 8-fold higher than wild type and heterozygous levels (Table 2). Follicle stimulating hormone (FSH) levels were not significantly different (Table 2). The observed ENERKI physiology is consistent with a lack of estrogen negative feedback at the pituitary, allowing for chronically elevated circulating levels of LH. The action of LH on the theca cells produced hypertrophied morphology and an overall increase in the levels of circulating estrogen and testosterone in the ENERKI mice. These studies confirm ERα ligand-induced signaling is critical for normal development of the female reproductive tract. The overall similarity of the ENERKI and αERKO[2] phenotype confirms ERα ligand-induced, but not ligand-independent, signaling is critical in female reproductive tract development.

Recent studies demonstrate ligand-independent signaling may play a dominant role in the non-reproductive organs. Studies are underway to examine the phenotype of several ENERKI non-reproductive tissues, including the bone and brain. In addition, ENERKI mice are currently being bred with ERE-luciferase (ERE-luc) mice. ERE-luc mice express a luciferase reporter that produces bioluminescence in tissues with ER transcriptional activity[3]. Therefore, ENERKI/ERE-luc mice can be used to determine where ligand-independent signaling occurs. If we confirm growth factors play a dominant role in non-reproductive tissues, these ligand-independent pathways may become important secondary targets for hormone replacement therapy.

c. Since 3-week-old wild type, heterozygous, and homozygous animals all had a rudimentary ductal mammary gland tree, 6-, 12-, and 20-week-old animals were examined. Mammary gland whole mounts of 6-week-old animals showed ENERKI females had a rudimentary underdeveloped epithelial ductal tree, while wild type and heterozygous females had a ductal tree extending to the lymph node and enlarged terminal end buds (Figure 5 A-C). In 12-week-old animals, wild type and heterozygous ducts filled the entire mammary fat pad and had extensive branching and alveolar budding, while homozygous mammary glands did not develop beyond a rudimentary epithelial ductal tree (Figure 5 D-F). This indicated the ENERKI mammary glands, like those of αERKO females[1], were unresponsive to estrogen. Western blots and IHC have confirmed equal ERα levels in the mammary gland. In the future, mRNA and protein expression levels for progesterone receptor (PR) will be quantified.

2. Determine the optimal DES dose for normal mammary gland development and fertility.

a. Immature mouse uterotrophic assays with E2 and DES were performed to confirm E2 would not stimulate the mutant G525L ERα receptor *in vivo* and DES would activate the receptor. For the uterotrophic assays, females of 18-21 days of age were subcutaneously injected with various doses of vehicle, E2 or DES for three consecutive days and sacrificed on the morning of the fourth day[4]. The uterine wet weight to body weight ratio was determined as a measure of uterine estrogenic response[4]. As anticipated, E2

increased uterine wet weights of wild type and heterozygous mice in a dose-dependent manner, but did not affect uterine wet weights of ENERKI mice (Figure 6A). DES also increased uterine wet weights of wild type and heterozygous mice in a dose-dependent manner (Figure 6B). Surprisingly, ENERKI mice only exhibited a slight increase in uterine wet weight in response to increasing concentrations of DES (Figure 6B). Wild type uterine wet weights were 6 and 4 times higher than ENERKI levels at 1000 and 10,000 ug/kg of DES, respectively (Figure 6B). ENERKI wet weights did not reach wild type and heterozygous levels until 100,000 ug/kg of DES was administered (Figure 6B). Since 100,000 ug/kg DES is an extremely high dose, we tested the hypothesis that DES priming may be required for a normal uterotrophic response in the ENERKI mice (e.g. for via upregulation of co-factors). Heterozygous females were treated with 10 ug/kg of DES during gestational days E9-birth and/or ENERKI pups were treated with a range of DES doses daily after birth. Neither treatment regime resulted in a significant uterotrophic response in the ENERKI animals. To determine whether long-term treatments were needed to induce a uterotrophic response, 4-week-old ENERKI females were treated every fourth day with 100 or 1000 ug/kg DES for a month. Although these animals did not exhibit a uterotrophic response, they did have significantly lower body weights, and gonadal fat pad and mammary gland weights than vehicle or E2 treated females. This demonstrates the mutant ERα is functional in the adipose tissue. Since DES treatments were not working well, we investigated if another ligand would bind the mutant G525L ERα *in vitro*. Fortunately, PPT treatments activated G525L ERα mutant transcription in a luciferase assay (Figure 2). PPT treatments also stimulated uterine wet weight increases in a dose-dependent manner in wild type, heterozygous, and ENERKI females (Figure 6C). Recent studies have demonstrated PPT is as efficacious as E2 in animals, but less potent[5]. Therefore, we were not surprised that high PPT doses were needed to stimulate a uterotrophic response in ENERKI animals. We are currently investigating if PPT priming will increase the ENERKI wet weight response in immature animals. Next, we will determine the optimal PPT dose and injection schedule to induce normal reproductive tract development in both the female and male ENERKI animals. Then, we will withdraw PPT at different time points to determine the impact of ligand-induced ERα activation during development.

.The remaining parts of task one and two will be completed in the future.

KEY RESEARCH ACCOMPLISHMENTS

- Estrogen non-responsive ERα knock-in (ENERKI) mice were generated.
- Body, gonadal fat pad, and mammary gland weights of ENERKI females were measured.
- The ENERKI female reproductive tract was extensively analyzed.
- LH, E2, and T serum levels were measured in 12-week-old animals.
- PPT was found to stimulate the G525L ERα mutant *in vivo*.

REPORTABLE OUTCOMES

Animal Model Generation:
ERα G525L knock-in mice

Oral Presentation:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in mice. Cancer Biology Retreat, Delevan, WI, May 2005.

Poster Presentation:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in mice. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, June 2005.

Oral and Poster Presentation:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in mice. Keystone Symposium on Nuclear Receptors: Steroid Sisters, Banff, Alberta, March 2006.

Paper:
Sinkevicius KW, Burdette JE, Woloszyn K, Temple KA, Wondisford FE, Korach KS, Woodruff TK, and Greene GL. Characterization of female estrogen non-responsive estrogen receptor alpha knock-in (ENERKI) mice. (in preparation).

CONCLUSIONS

Phenotypic analysis of the ENERKI mice revealed ligand-induced ERα signaling is crucial in female adiposity regulation and reproductive tract development. Uterotrophic assays with epidermal growth factor (EGF) and insulin-like growth factor (IGF) will be important in determining the role of cross-talk between ERα and growth hormone signaling pathways *in vivo*. Since ligand-independent signaling is hypothesized to be essential in non-reproductive tissues, analysis of the bone and brain phenotype should be particularly interesting. The mechanisms of ERα in mammary gland development and tumorigenesis remain poorly understood partly due to the lack of suitable animal models to study ERα action *in vivo*. Phenotypic analysis and PPT treatment of ENERKI mice will increase our knowledge about the ligand-induced and ligand-independent signaling mechanisms of ERα. The ENERKI mice will be used to study the genesis and progression of hormone dependent mammary cancers and this information should facilitate the development of novel therapies for the treatment and prevention of breast cancer.

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Table 1. Female body, gonadal fat pad, and mammary gland weights

12 Week Mice	Body Weight (grams)	Gonadal Fat Pad (% Body Weight)	Mammary Gland (% Body Weight)
Regular Diet			
WT, n=10	21.4 (+/- 0.9)	2.4 (+/- 0.3)	1.1 (+/- 0.1)
HET, n=11	21.1 (+/- 0.9)	1.9 (+/- 0.3)	1.2 (+/- 0.1)
ENERKI, n=10	25.4 (+/- 1.1)[a]	2.1 (+/- 0.2)	1.1 (+/- 0.1)
Soy-Free Diet			
WT, n=11	20.6 (+/- 0.5)	1.9 (+/- 0.2)	0.9 (+/- 0.1)
HET, n=9	21.8 (+/- 1.0)	3.2 (+/- 0.5)	1.1 (+/- 0.1)
ENERKI, n=10	25.4 (+/- 1.2)[a]	3.6 (+/- 0.5)[b]	1.3 (+/- 0.1)[b]

Data expressed as mean (+/- SEM).
[a] ENERKI value significantly different from WT and HET value ($p < 0.01$).
[b] ENERKI value significantly different from WT value ($p < 0.01$).

Table 2. Female serum hormone levels

Mice	LH (ng/mL)	FSH (ng/mL)	E2 (pg/mL)	T (ng/mL)
WT, n=6-14	0.31 (+/- 0.05)	3.80 (+/- 0.36)	9.74 (+/- 2.11)	0.02 (+/- 0.00)
HET, n=10-14	0.38 (+/- 0.07)	4.47 (+/- 0.70)	16.36 (+/- 2.00)	0.03 (+/- 0.01)
HOM, n=8-14	3.72 (+/- 0.42)[a]	3.43 (+/- 0.22)	38.22 (+/- 7.19)[a]	0.88 (+/- 0.22)[a]

Data expressed as mean (+/- SEM).
[a] ENERKI value significantly different from WT and HET value ($p < 0.01$).

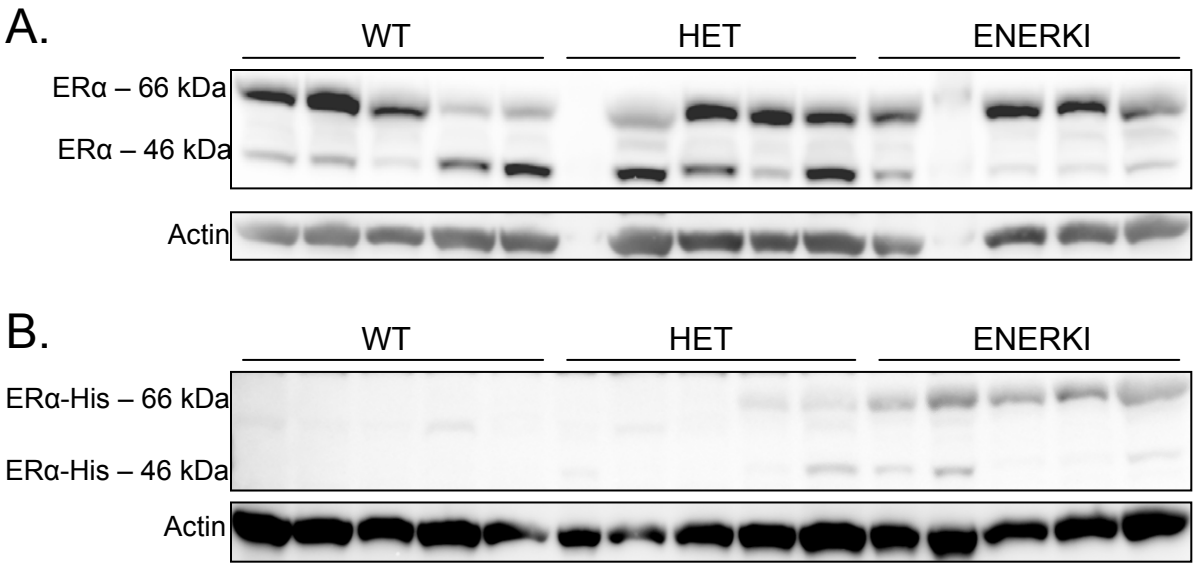

Figure 1. Western blot analysis of uterine protein extracts.
A: Western blot analysis of uterine protein extracts using an antibody to ERα.
B: Western blot analysis of uterine protein extracts using an antibody to 6xHis-tag.

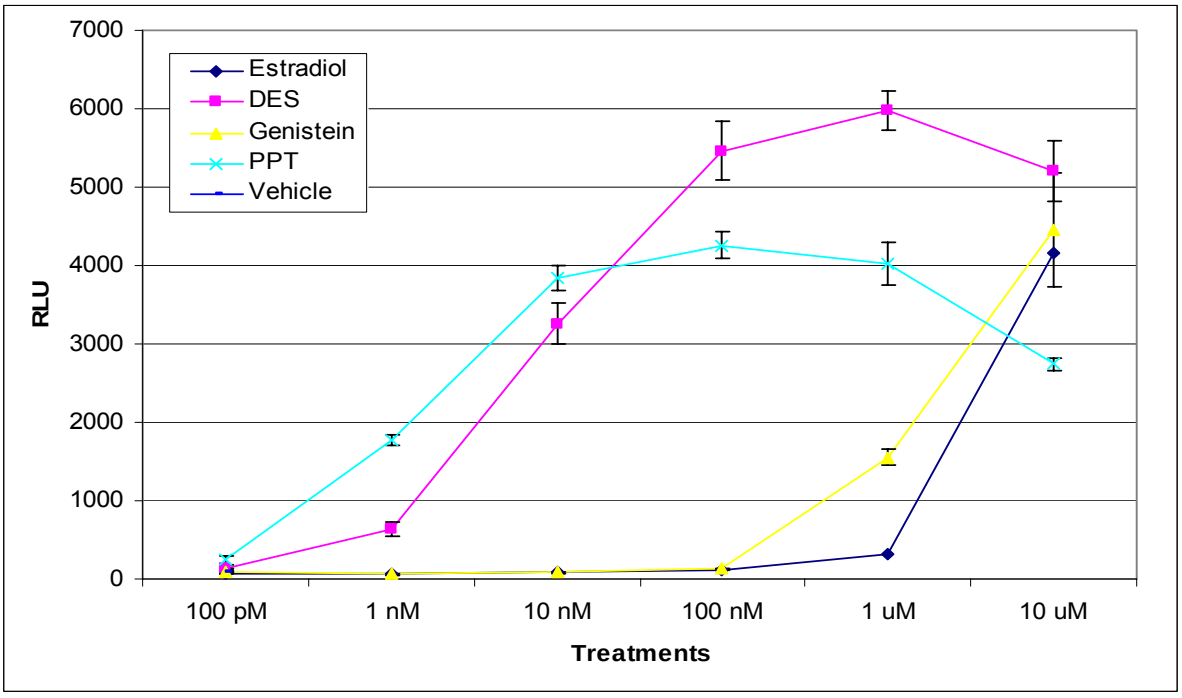

Figure 2. Transcriptional activation of mutant G525L ERα.
Luciferase assays with Ishikawa cells, cotransfected with mutant G525L ERα and an ERE-reporter, showed transcription was not stimulated by low concentrations of E2, but was stimulated by DES, genistein, or PPT. Luciferase activity was normalized for transfection efficiency using β-galactosidase as an internal control

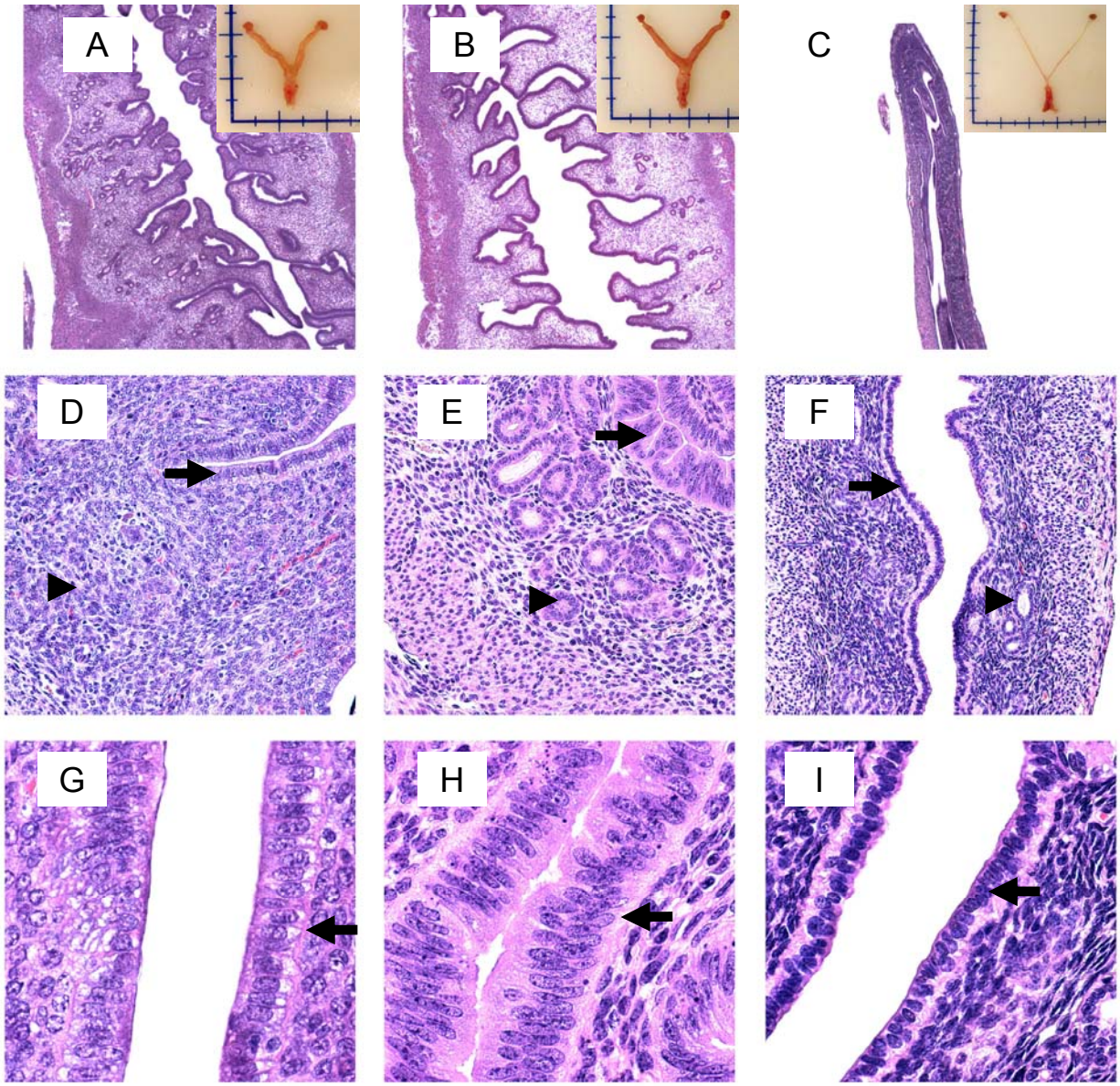

Figure 3. Uterine histology of representative mice.
Uterine tissue H&E staining from 12-week-old wild type (A,D,G), heterozygous (B,E,H), and ENERKI (C,F,I) mice.
A-C: Wild type (A) and heterozygous (B) uteri developed normally, but ENERKI (C) tissues did not exhibit an increase in uterine wet weight and were immature and hypoplastic (10x). Insert: reproductive tract.
D-F: Wild type (D) and heterozygous (E) uterine tissue had a normal luminal epithelium (black arrow), ductal epithelium (black arrowhead), and stroma, while ENERKI (F) uterine tissue displayed a lack of estrogenization of the luminal and glandular epithelium (20x).
G-I: Large secretory vacuoles and enlarged cytoplasm were present in the wild type (G) and heterozygous (H) luminal epithelium (black arrows point to the nuclei of luminal epithelium), respectively, while the ENERKI (I) epithelium lacked vacuoles (40x).

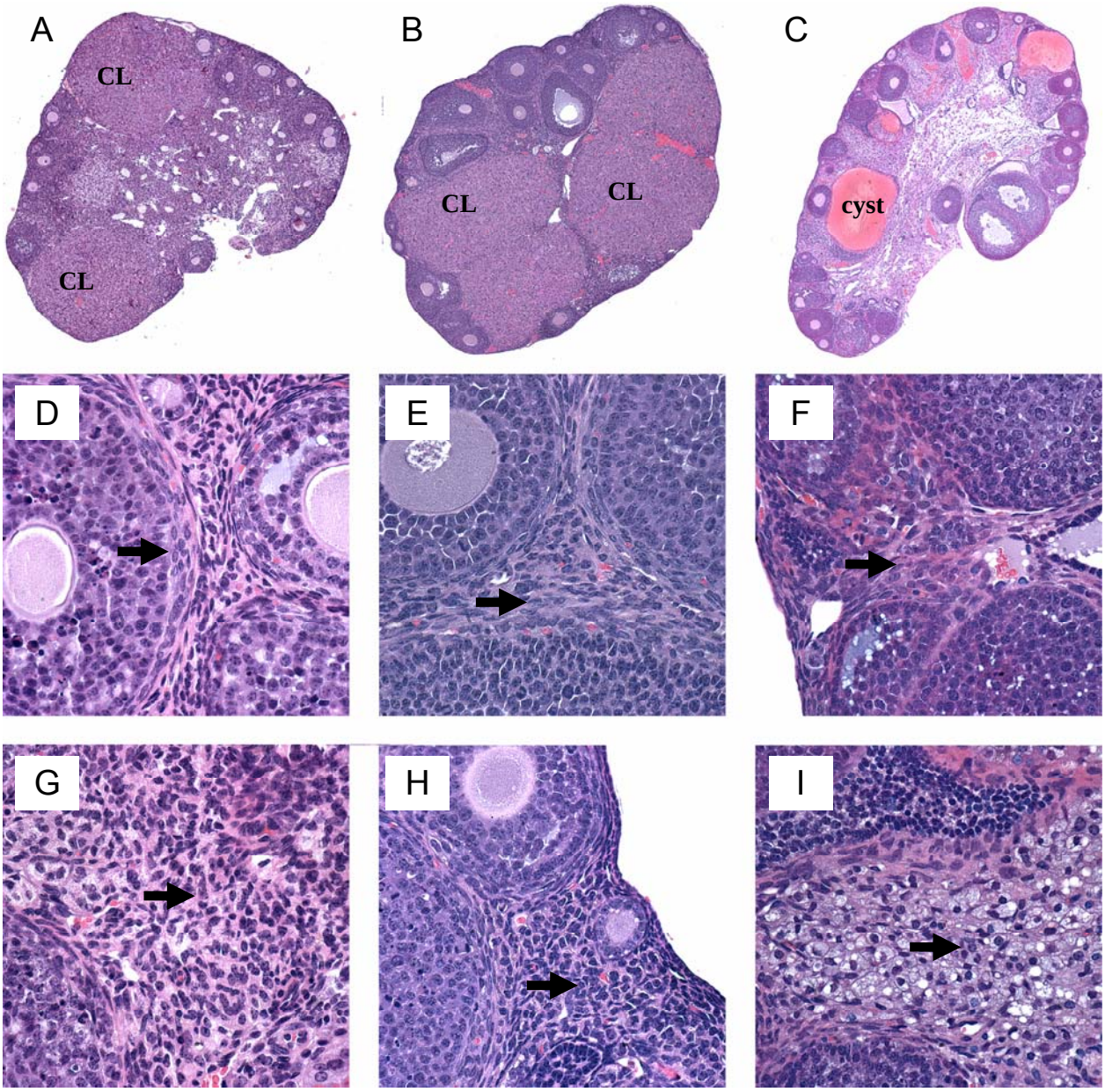

Figure 4. Ovarian histology of representative mice.
Ovarian tissue H&E staining from 12-week-old wild type (A,D,G), heterozygous (B,E,H), and ENERKI (C,F,I) mice.
A-C: Wild type (A) and heterozygous (B) ovaries contained many corporta lutea and healthy follicles, while ENERKI (C) ovaries had no corpora lutea (CL) and the majority contained large hemorrhagic cysts (10x).
D-F: Theca cell layers (black arrow) were normal in wild type (D) ovaries, but hypertrophied in heterozygous (E) and ENERKI (F) ovaries (40X).
G-I: Stroma (black arrow) was normal in the wild-type (G) and heterozygous (H) tissues, while the ENERKI stroma (I) possessed luteinized interstitial hyperplasia (40X).

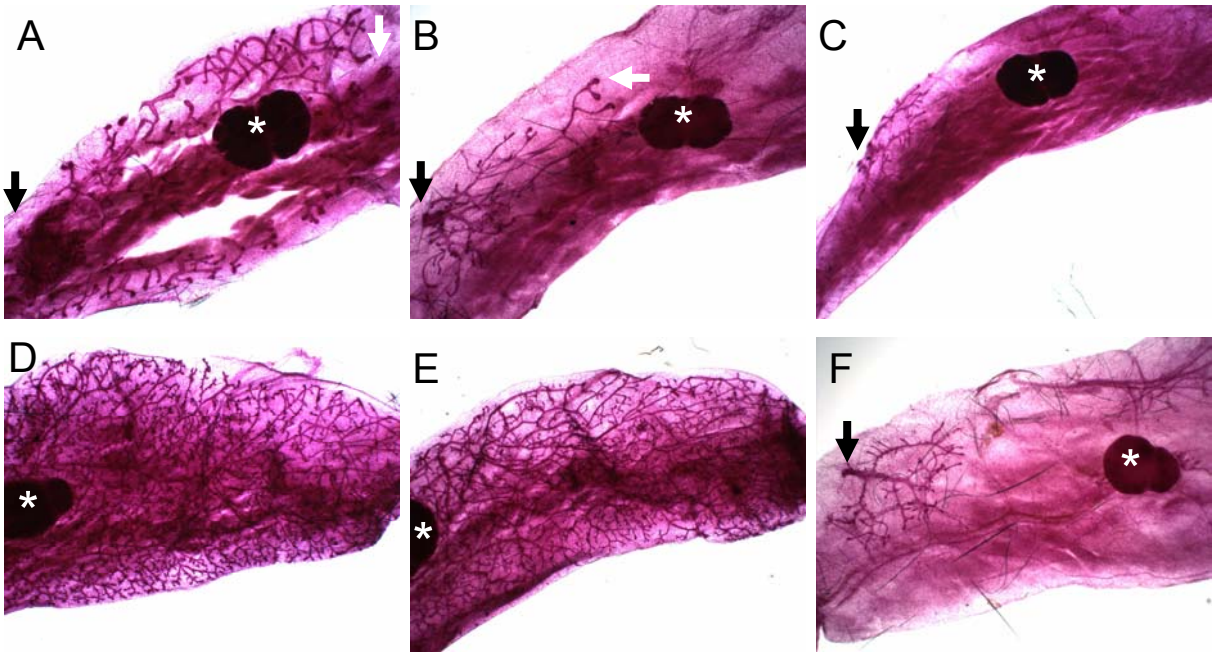

Figure 5. Mammary gland whole mounts of representative mice.
A-C: Mammary gland whole mounts from 6-week-old wild type (A), heterozygous (B), and ENERKI (C) mice (1.25x). Wild type and heterozygous ductal trees extended to the lymph node (white asterisk) and had enlarged terminal end buds (white arrow), while homozygous mammary glands had a rudimentary epithelial ductal tree. Nipple location is indicated by the black arrow. **D-F:** Mammary gland whole mounts from 12-week-old wild type (D), heterozygous (E) and ENERKI (F) mice (1.25x). Wild type and heterozygous ducts filled the entire mammary fat pad and had extensive branching and alveolar budding, while ENERKI mammary glands did not develop beyond a rudimentary epithelial ductal tree.

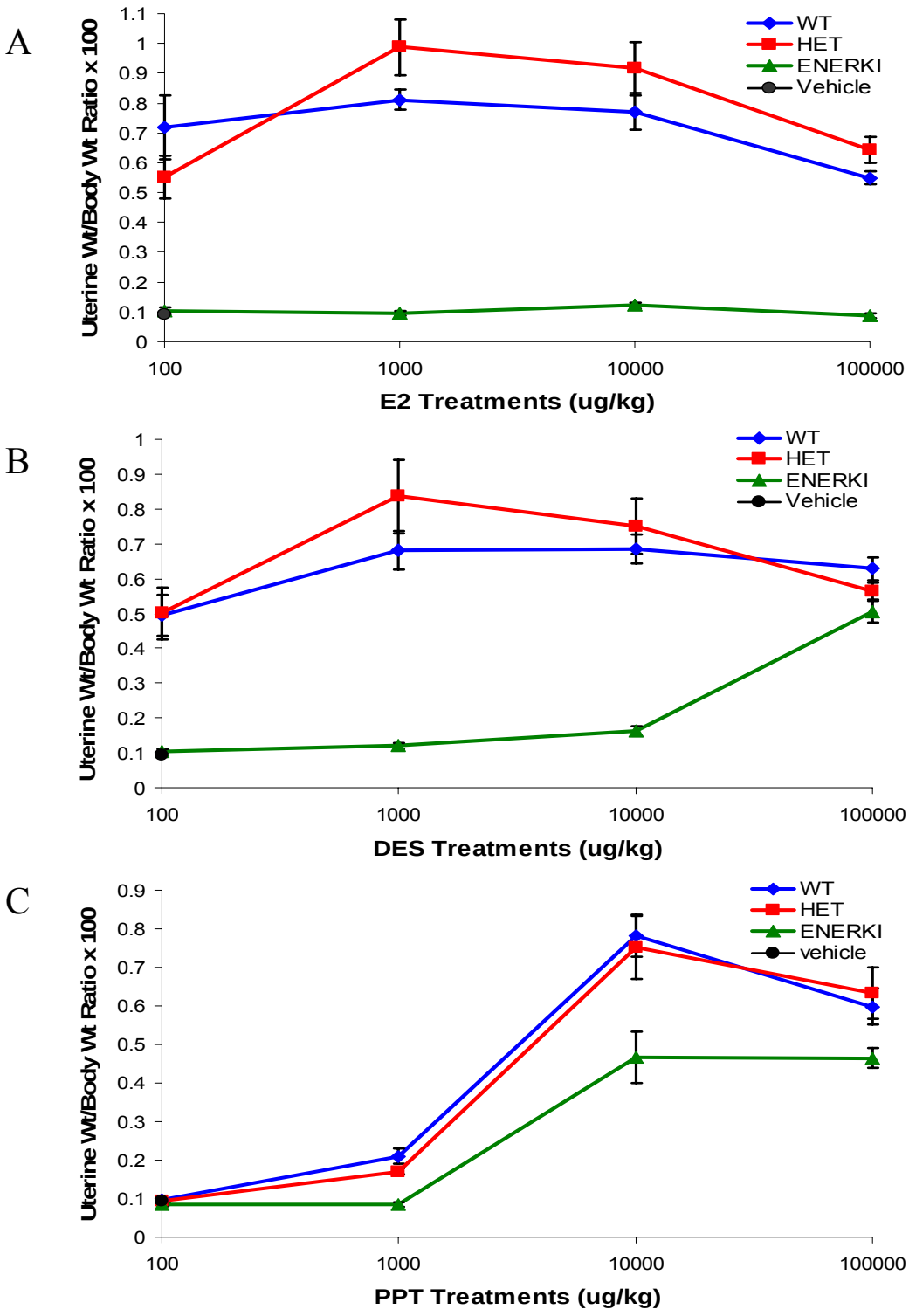

Figure 6. Uterotrophic activity of E2, DES, and PPT.
Immature wild type, heterozygous, or ENERKI female mice were subcutaneously injected with the indicated doses of vehicle, E2 (A), DES (B), or PPT (C) for three consecutive days. Uterine wet weight was measured on the fourth day. Values represent mean +/- SEM with 4-5 animals per group. WT and HET levels were not statistically different at any point. ENERKI values were significantly different ($p<0.01$) from WT and HET values for all treatments (A), or for 100, 1000, and 10,000 ug/kg (B & C).